

Candidate Adaptor Protein CED-6 Promotes the Engulfment of Apoptotic Cells in *C. elegans*

Qiong A. Liu*† and Michael O. Hengartner*†

*Cold Spring Harbor Laboratory

1 Bungtown Road

Cold Spring Harbor, New York 11724

†Department of Molecular Genetics and Microbiology
SUNY at Stony Brook

Stony Brook, New York 11794

Summary

The rapid engulfment (phagocytosis) of cells undergoing programmed cell death (apoptosis) is a fundamental biological process that is not well understood. Here we report the cloning and functional characterization of *ced-6*, a gene specifically required for the engulfment of apoptotic cells in the nematode *C. elegans*. The CED-6 protein contains a phosphotyrosine binding domain at its N terminus and a proline/serine-rich region in its C-terminal half. Genetic mosaic analysis demonstrates that *ced-6* acts within engulfing cells. We also show that *ced-6* can promote the engulfment of cells at both early and late stages of apoptosis. Our data suggest that CED-6 is an adaptor molecule acting in a signal transduction pathway that specifically mediates the recognition and engulfment of apoptotic cells.

Introduction

Phagocytosis, or engulfment, is a specialized form of endocytosis through which eukaryotes take up very large particles, or even whole cells. It is a fundamental biological process conserved from single-cell organisms such as amoebae to mammals (Metchnikoff, 1891). Initially used for the dual purpose of feeding and defense, phagocytosis evolved, following the emergence of mesoderm, into a mechanism used to protect the host against invading organisms and to clear up foreign particles and cell debris (Metchnikoff, 1891). Recently, the significance of phagocytosis has been extended due to its role in eliminating cells undergoing programmed cell death (apoptosis).

Programmed cell death is usually divided into two distinct sequential processes—cell killing and the removal of dead cells—and these two events are very closely linked. In vivo, cells that present an apoptotic morphology are usually found within other cells (Wyllie et al., 1980; Lockshin, 1981; Robertson and Thomson, 1982; Hedgecock et al., 1983; Duvall and Wyllie, 1986; Ellis et al., 1991). Engulfment in *C. elegans* is also a swift and efficient process; dying cells are engulfed and completely removed by their neighboring cells within an hour (Sulston and Horvitz, 1977; Robertson and Thomson, 1982). Rapid engulfment of apoptotic cells is important, as it prevents dying cells from releasing potentially

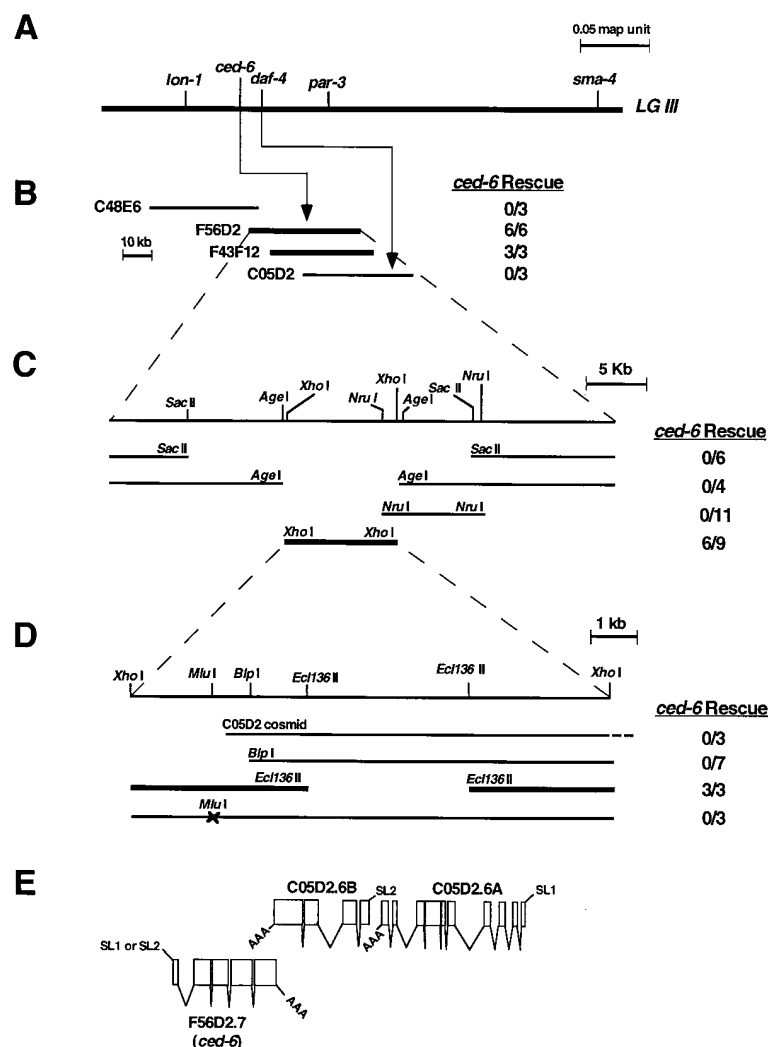
harmful contents during their lysis, which could damage surrounding tissue and result in an inflammatory response (Duvall et al., 1985; Savill et al., 1989, 1993; Grigg et al., 1991).

The nematode *C. elegans* has been used extensively for the study of programmed cell death (reviewed by Hengartner, 1997). Genetic studies have identified over a dozen genes that function in the regulation and execution of apoptosis in *C. elegans*. Six genes—*ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7*, and *ced-10*—function in the engulfment of all dying cells (Hedgecock et al., 1983; Ellis et al., 1991; Horvitz et al., 1994). In animals mutant for any one of these genes, many apoptotic cells fail to be engulfed and persist for many hours as highly refractile disks that can be readily identified under differential interference contrast (DIC) optics (Hedgecock et al., 1983; Ellis et al., 1991). None of the six engulfment genes is absolutely essential for engulfment, as many dying cells are still properly removed in these mutants. Genetic analysis of various double mutants suggested that these six genes might form two partially redundant groups, one being comprised of *ced-1*, *ced-6*, and *ced-7*; the other, of *ced-2*, *ced-5*, and *ced-10* (Ellis et al., 1991). The number of persistent cell corpses is increased dramatically in double mutants crossing groups, but not in those within the same group. Understanding how these genes are involved in regulating engulfment will require the elucidation of their molecular nature.

In other species, several candidate apoptotic receptors have been identified over the past few years; these include the ATP-binding cassette transporter ABC1 (Luciani and Chimini, 1996), adhesion molecules such as the vitronectin receptor (Savill et al., 1990) and CD36 (Asch et al., 1987; Savill et al., 1992; Ren et al., 1995), *Drosophila croquemort* (Franc et al., 1996), class A scavenger receptors (Platt et al., 1996), lectins (Duvall et al., 1985), and a predicted receptor that can recognize phosphatidylserine on the outer leaflet of apoptotic cells (Fadok et al., 1992a, 1992b). However, we currently know little about the molecules used by engulfing cells to transduce signals from surface receptors to the cytoskeleton, or how these molecules regulate the local cytoplasmic rearrangements and dynamic extensions that are required for phagocytosis (Savill et al., 1993). A genetic analysis of engulfment in *C. elegans* might identify genes involved in these processes. Indeed, Wu and Horvitz (1998a) recently showed that *C. elegans* CED-5 is homologous to human Dock180 and might regulate cytoskeleton rearrangement during engulfment.

We report here the positional cloning and functional characterization of the *C. elegans* gene *ced-6*. We show that the CED-6 protein contains a phosphotyrosine-binding domain and several potential SH3-binding sites. We demonstrate that CED-6 acts within engulfing cells and functions to promote the removal of both early and persistent cell corpses. Overexpression of *ced-6* can partially suppress the engulfment defect of both *ced-1* and *ced-7*, suggesting that *ced-6* might function downstream of these two genes. We conclude that CED-6 acts as an adaptor molecule in a signal transduction

† To whom correspondence should be addressed.

Figure 1. The *ced-6* Locus

Approximate position of *ced-6* on the genetic (A) and physical (B) maps was determined using data from previously published genetic crosses (Ellis et al., 1991) and the position of the *daf-4* gene on the physical map (Estevez et al., 1993). Cosmids (B) or subcloned DNA fragments (C and D) from this region were used to generate transgenic lines that were tested for *ced-6(n1813)* rescue. Lines that gave 3-fold embryos with few or no cell corpses were considered to be rescued. Rescue data is given as number of rescuing lines/total number of transgenic lines tested. Only four of the 13 cosmids tested are shown in (B). Both F56D2 and C05D2 were sequenced by the *C. elegans* Genome Project. Restriction maps of the rescuing F56D2 cosmid and the *Xho I* subclone are shown in (C) and (D), respectively. Transcripts identified on the *Xho I* fragment are shown in (E). Rescuing clones are shown in bold. X, frameshift mutation introduced into exon 3 of F56D2.7. Boxes, exons; AAA, poly(A) tail; SL1 and SL2, trans-spliced leader sequences.

pathway that mediates the engulfment of apoptotic cells in *C. elegans*.

Results

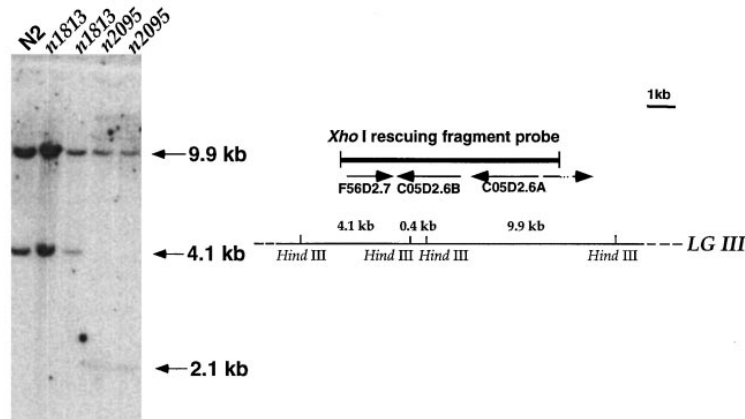
Molecular Cloning of *ced-6*

Previous genetic mapping experiments by Ellis and colleagues (Ellis et al., 1991) have suggested that the *ced-6* locus lies near *daf-4* (Estevez et al., 1993) on chromosome III (Figure 1A). The region around *daf-4* has been mostly sequenced by the *C. elegans* genome sequence consortium (Wilson et al., 1994). Using the germ-line transformation method (Mello and Fire, 1995), we tested cosmids from this region for their ability to rescue the engulfment defect of *ced-6(n1813)*. We found that two overlapping cosmids, F56D2 and F43F12, were each individually able to rescue the *ced-6* engulfment defect, whereas the flanking cosmids C48E6 and C05D2 failed to do so (Figure 1B). By generating and testing various F56D2 deletion derivatives and subclones, we further restricted the *ced-6* rescuing activity to a 10 kb *Xho I* fragment present in both F56D2 and F43F12 (Figures 1C and 1D).

The *ced-6* Locus

Using a combination of RT-PCR and cDNA library screening (see below), we identified three genes within the 10 kb *Xho I* fragment (Figure 1E). We found that the deletion of most of the C05D2.6A/B operon had no deleterious effect on *ced-6* rescue, whereas the introduction of a frameshift mutation predicted to disrupt the F56D2.7 reading frame abolished the fragment's rescuing activity (Figure 1D). To exclude the possibility that F56D2.7 might be a multicopy suppressor of *ced-6*, rather than *ced-6* itself, we analyzed the two known mutant *ced-6* alleles, *n1813* and *n2095*, for any nucleotide changes within this region. Southern blot analysis revealed an allele-specific restriction fragment length polymorphism affecting F56D2.7 in *ced-6(n2095)* mutants (Figure 2A). We also identified a single nucleotide deletion in exon 4 of F56D2.7 in *ced-6(n1813)*; this frameshift mutation would lead to the production of a truncated protein (Figure 2B). Taken together, the genomic rescue and mutation data strongly suggest that F56D2.7 corresponds to *ced-6*. This conclusion is also supported by the ability of F56D2.7 cDNA to rescue the *ced-6* mutant phenotype (see below).

A



B

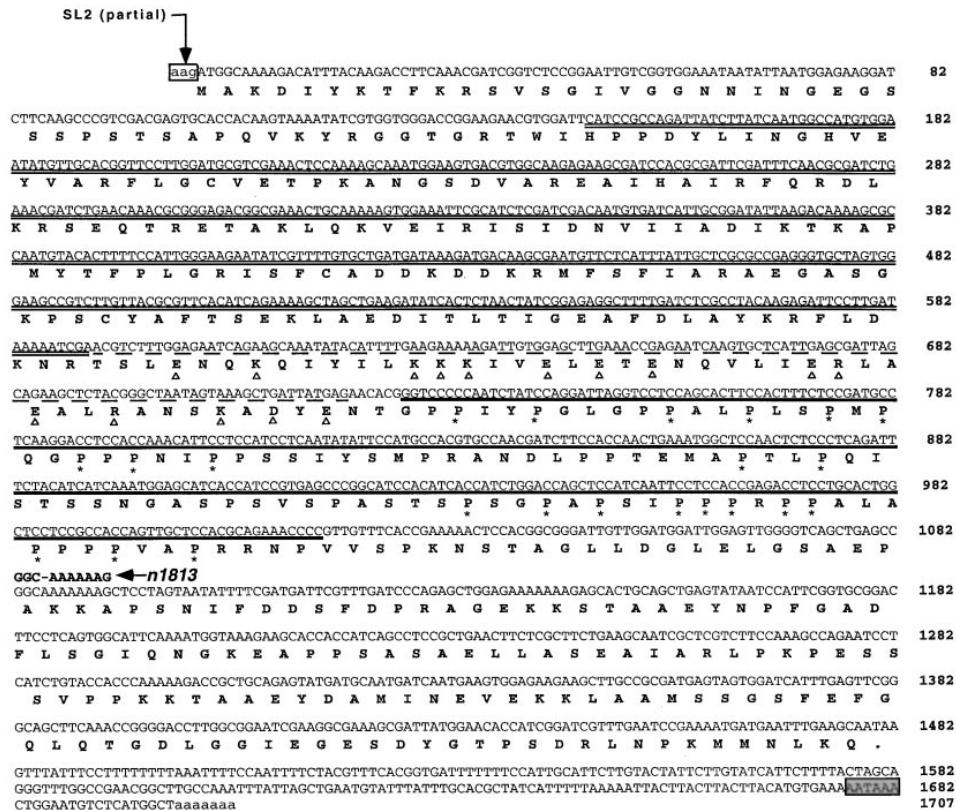


Figure 2. F56D2.7 Encodes CED-6

(A) Allele-specific polymorphism in *ced-6(n2095)* affects F56D2.7. A Southern blot of HindIII-digested genomic DNA isolated from wild-type, *ced-6(n1813)*, and *ced-6(n2095)* mutants was probed with a ³²P-labeled XhoI fragment. HindIII genomic fragments that hybridize to the probe are shown on the right. In *ced-6(n2095)*, the 4.1 kb fragment is missing, and a novel 2.1 kb fragment is apparent.

(B) Sequence of full-length F56D2.7 cDNA clone pLQ33.1 and its predicted translation product. F56D2.7 can be *trans*-spliced to either SL1 or SL2; clone pLQ33.1 contains the last three nucleotides (AAG) of SL2 (open box). Shaded box, putative polyadenylation signal. The single base-pair deletion identified in *ced-6(n1813)* is shown above the cDNA sequence. Protein domains are marked as follows: PTB domain, double underline; proline/serine-rich region, single underline; charged region, dashed underline. Stars mark the prolines in the PxxP signature sequences; triangles mark charged residues within the charged region. The *ced-6* cDNA sequence has been submitted to GenBank under accession number AF061513.

Identification of *ced-6* Transcripts

To confirm the predicted intron/exon structure for *ced-6*, we screened a mixed-stage cDNA library and identified 10 clones corresponding to the *ced-6* gene. Several of these contained splice leader SL2 sequences at their 5' end, suggesting that *ced-6* might be a downstream gene in a multigene operon (Blumenthal and Steward, 1997). To test this hypothesis, we performed RT-PCR on mixed-stage RNA using either SL1 or SL2 splice leader sequences as primers for the PCR step. Interestingly, products could be amplified using both primers, indicating that both SL1 and SL2 splice leaders can be found at the 5' end of *ced-6* transcripts (Figure 2B, data not shown). We suspect, but have not proved, that the upstream gene in the *ced-6* operon is the predicted gene F56D2.1. The existence of SL1 *trans*-spliced mRNA suggests that *ced-6* might also be transcribed from a second downstream promoter, independently of the upstream gene (Blumenthal and Steward, 1997), and could possibly explain why the XhoI fragment can rescue *ced-6* mutants even though it does not contain the whole *ced-6* operon.

CED-6 Protein Contains a Phosphotyrosine-Binding (PTB) Domain and a Proline/Serine-Rich Region

The full-length *ced-6* cDNA is predicted to code for a 492-amino acid protein (Figure 2B). A search of public sequence databases with the predicted CED-6 sequence revealed that the N-terminal half of CED-6 contains a putative phosphotyrosine-binding (PTB) domain. PTB domains can recognize phosphorylated tyrosine residues within an NPXphosphoY consensus sequence (Kavanaugh et al., 1995; Songyang et al., 1995; Zhou et al., 1995). The PTB domain is similar to the SH2 domain in function, but distinct in structure (Blaikie et al., 1994; Kavanaugh and Williams, 1994; Zhou et al., 1995; Pawson and Scott, 1997). We have aligned the CED-6 PTB domain with PTB domains found in a number of other proteins, using sequence similarities and the known NMR structure of the PTB domain of Shc (SH2-containing) protein (Zhou et al., 1995) as guidelines (Figure 3A). CED-6 PTB domain shares 20%–30% identity with PTB domains of identified proteins (Figure 3B). The PTB domain of Shc contains two anti-parallel β sheets and three α helices (Zhou et al., 1995); secondary structure prediction programs suggest that most of these structural elements also exist in the CED-6 PTB domain.

Within its C-terminal half, CED-6 contains a proline/serine rich region (Figures 2B and 3B) that contain several PxxP sequence signatures (Figure 2B). PxxP sequences have been shown to promote, in the right sequence context, interaction with an SH3 domain (Ren et al., 1993; Yu et al., 1994; Grabs et al., 1997). Between the PTB and proline-rich regions is a short stretch rich in charged residues (35% charged amino acids over 43 amino acids). A highly charged region is also found in several other PTB domain-containing proteins, including mouse p96, Shc, and *C. elegans* M110.5 (Figure 3B).

CED-6 Might Be Conserved among Species

In addition to its similarity to known proteins, CED-6 also showed significant sequence similarity to the predicted

translation products of a number of expressed sequence tags (ESTs), including three overlapping human EST clones (y161g04, hbc3123, and EST178829), *Drosophila* EST clone LD09044, and *C. briggsae* EST clone pk02f09 (Figures 3A and 3B and data not shown). Interestingly, the sequence similarity in the PTB domain between CED-6 and the above-mentioned EST clones is much higher than between CED-6 and any previously characterized protein. For the human and *C. briggsae* EST clones, the sequence conservation also extends beyond the limits of the PTB domain (Figures 3B and 3C). An evolutionary relationship analysis based on an alignment of PTB domains showed that CED-6 formed a distinct subgroup with the human, *Drosophila*, and *C. briggsae* EST clones, suggesting that these cDNAs might encode orthologs of *C. elegans* CED-6 (Figure 3C).

ced-6 Promotes the Engulfment of Dying Embryonic and Germ Cells

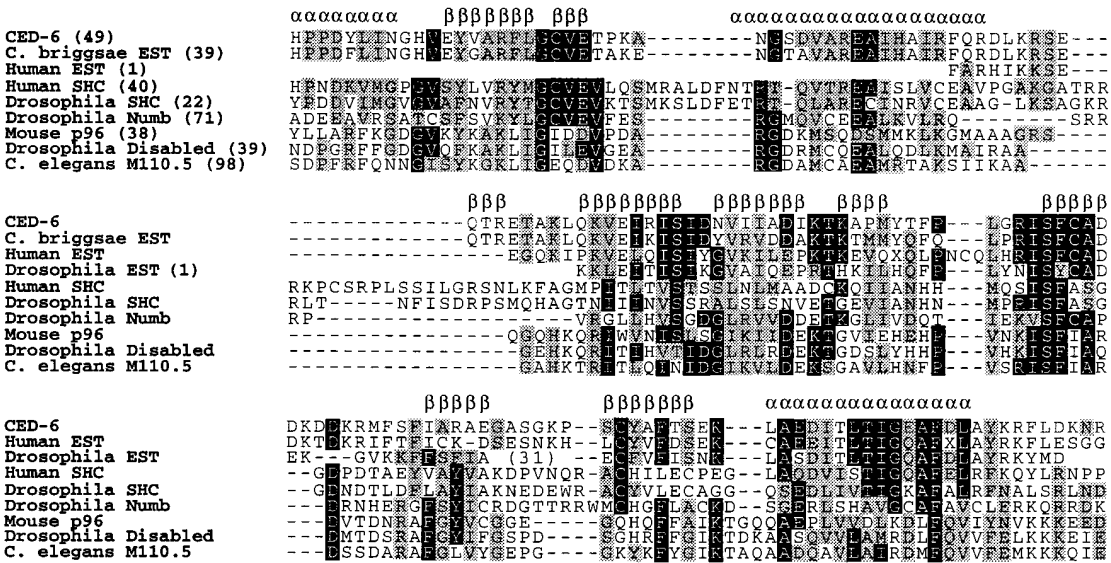
To unambiguously demonstrate that F56D2.7 corresponds to *ced-6*, we tested whether the full-length F56D2.7 cDNA can rescue the engulfment defect of *ced-6* mutants. Towards this end, we generated transgenic animals carrying a F56D2.7 cDNA under the control of the *C. elegans* heat shock promoters *hsp-16.2* and *hsp-16.48* (see Experimental Procedures). Used in combination, these two promoters drive expression in almost all somatic cells (Stringham et al., 1992), including cells that undergo programmed cell death (Hengartner and Horvitz, 1994) and cells that normally engulf the dying cells (Robertson and Thomson, 1982). To test for rescue, we exposed embryos laid by transgenic mothers to a brief heat shock pulse just prior to the appearance of the first developmental cell deaths and scored for the presence of persistent corpses in the head region of heat-shocked animals shortly after hatching (Figure 4A). As expected, overexpression of F56D2.7 cDNA significantly reduced the number of persistent cell corpses visible in *ced-6* mutants (Figure 4B), confirming that F56D2.7 is the relevant gene affected by *ced-6(n1813)* and *ced-6(n2095)* mutations.

We also tested the ability of F56D2.7 cDNA to rescue the engulfment defect observed in the adult hermaphrodite germ line (Figure 4C). We exposed young adult hermaphrodites to a brief heat shock pulse shortly after the appearance of germ cell death and followed them over the next 60 hr to assess the presence of persistent germ cell corpses. Most heat-shocked animals had few germ cell corpses if any, indicating that overexpression of *ced-6* can also rescue the germ cell engulfment defect of *ced-6* mutants.

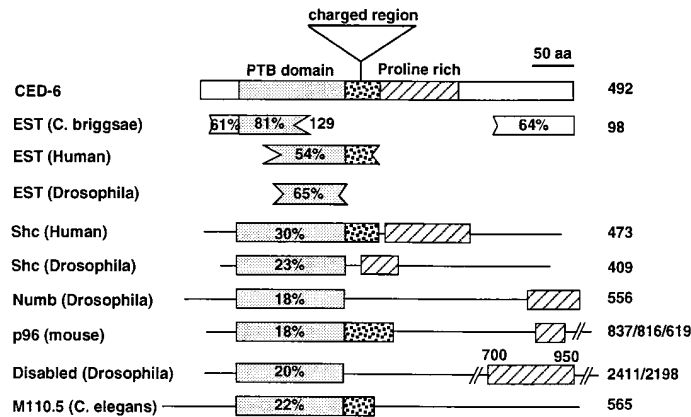
ced-6 Promotes the Engulfment of Persistent Cell Corpses

Recognition and engulfment of apoptotic cells is a very early event in *C. elegans* programmed cell death (Robertson and Thomson, 1982). In *ced-6* mutants many cells initially fail to be engulfed; these persistent cell corpses, however, eventually disappear over time (Ellis et al., 1991). The ultimate fate of these cell corpses is quite variable: while at least some of them appear to undergo

A



B



C

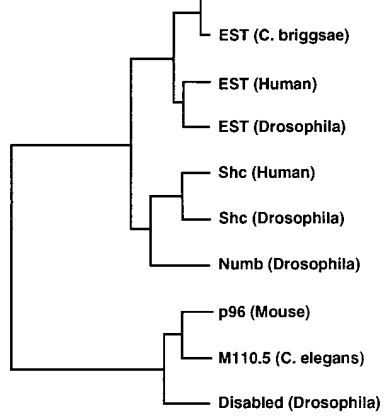


Figure 3. CED-6 Contains a Phosphotyrosine-Binding Domain

(A) Alignment of the CED-6 PTB domain with other PTB domains. The alignment is based on sequence similarities and the published NMR structure of the Shc PTB domain (Zhou et al., 1995). Black and gray boxes indicate identical and similar residues (shared by ≥50% of sequences shown), respectively. For this purpose, the following sets of amino acids were considered similar: G, A, S, T; E, D, Q, N; R, K, H; V, M, L, I; F, Y, W; C. α and β, α helices and β sheets identified in the Shc NMR structure. Numbers in parentheses, residue number of first amino acid shown for each protein.

(B) Structural domains in CED-6 and other PTB-containing proteins. Proline-rich, charged, and other domains of interest are highlighted. Percent identity of each PTB domain to the CED-6 PTB domain are shown within the PTB domain boxes.

(C) Evolutionary conservation among PTB domains. The alignment shown in (A) was used to develop an evolutionary relation tree. CED-6 clusters with a group of uncharacterized ESTs, whereas another *C. elegans* PTB domain protein, M110.5, clusters with p96 and disabled.

secondary necrosis and lysis, others are belatedly recognized and phagocytosed. To determine whether the *ced-6* pathway can promote the removal of late apoptotic corpses, we again used the *hs::ced-6* transgenic strains, but this time heat shocked the embryos 3 hr before hatching. By this time, most apoptotic cells in embryos have been dead for approximately 5 hr (Figure 4A). We found that the number of cell corpses present in young hatchlings was significantly lower than in the

controls (Figure 4B; $p < 0.0002$, unpaired Student's *t* test). We also tested if overexpression of *ced-6* could promote the engulfment of persistent germ cell corpses (Figure 4D). We found that heat shocking adult *hs::ced-6* transgenic animals dramatically reduced the number of germ cell corpses, usually down to wild-type levels. This effect was independent of the age of the animal or the number of persistent germ cell corpses present prior to heat shock. Thus, overexpression of *ced-6* can promote

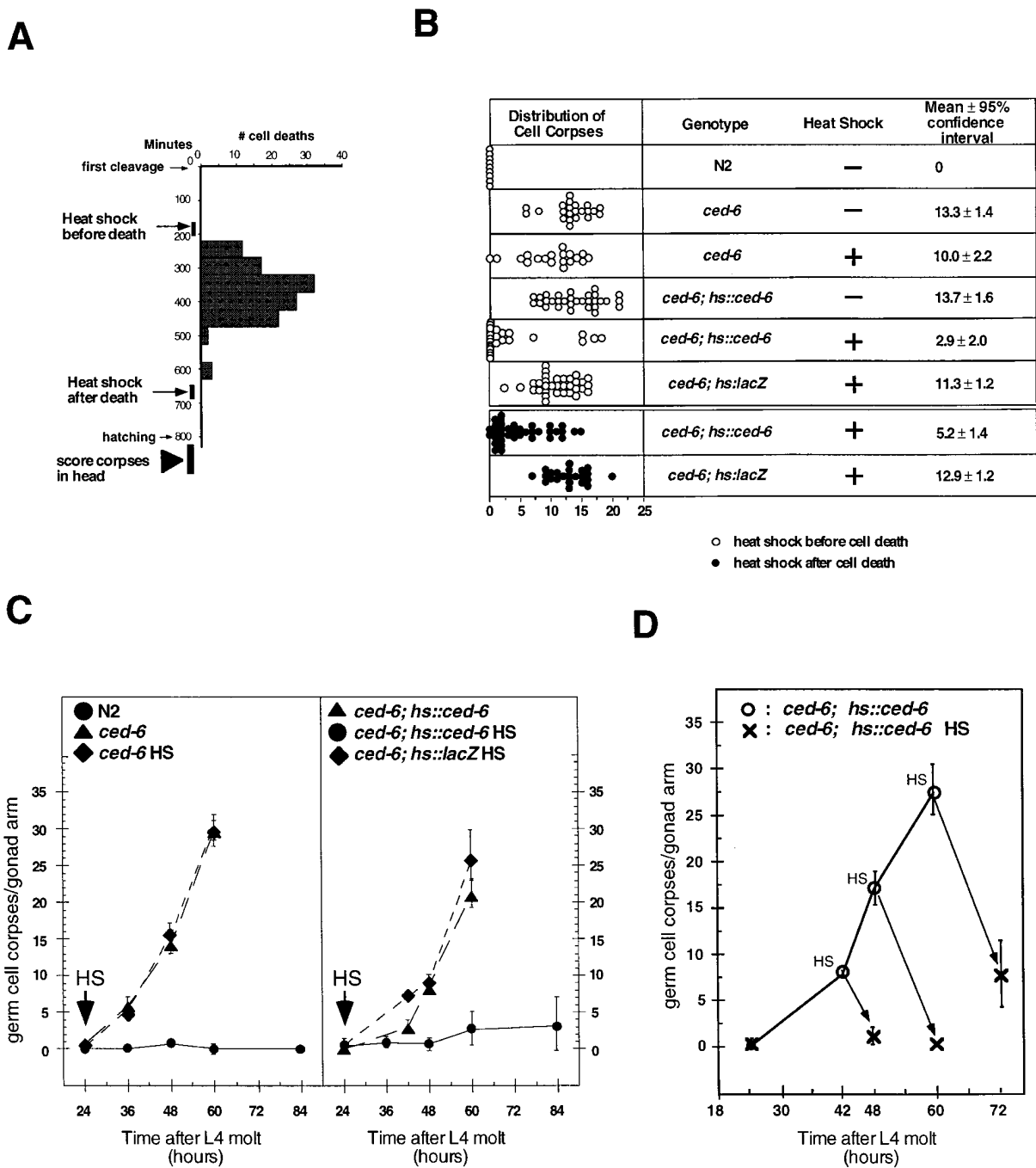


Figure 4. Overexpression of *ced-6* Promotes the Engulfment of Both Early and Persistent Cell Corpses

(A) Cell death during embryonic development. Embryonic development was divided into 50 min intervals, and the number of cells dying in each interval plotted as a function of developmental age. Embryos were heat shocked (arrows) either prior to or well after the main wave of embryonic cell death, and scored for the number of persistent cell corpses in the head region shortly after hatching (arrowhead). The experiments shown were performed with *ced-6(n1813)*.

(B) Overexpression of *ced-6* promotes the engulfment of both early and persistent embryonic cell corpses. Embryos laid by transgenic mothers were heat-shocked either before (open symbols) or after (filled symbols) the wave of embryonic cell death (see [A]), and scored for number of persistent cell corpses shortly after hatching.

(C) Overexpression of *ced-6* rescues the germ cell engulfment defect. Transgenic animals were heat shocked 24 hr after the L4/adult molt (arrow), and germ cell corpses were scored over the next 60 hr. In (C) and (D), symbols represent means \pm 95% confidence interval.

(D) Overexpression of *ced-6* DNA promotes the engulfment of persistent germ cell corpses. Adult transgenic animals were submitted to heat shock at various times after the L4/adult molt (arrows), and germ cell corpses counted 12 hr after heat shock.

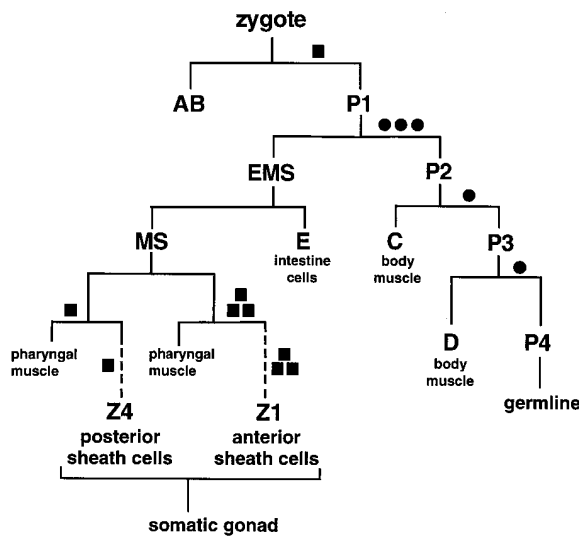


Figure 5. *ced-6* Acts within the Engulfing Cells
Genetic mosaic analysis was used to determine the focus of *ced-6* action in the engulfment of apoptotic germ cells by the surrounding sheath cells. The lineages that generate germ cells (which die) and the gonadal sheath cells (which engulf the dying germ cells) are shown. Mosaic animals were identified as described in Experimental Procedures, and the exact position of the duplication loss was determined by scoring related cells for the cell-autonomous marker gene *ncl-1*. Gonad arms of mosaic animals were either completely wild-type for engulfment, or as strongly defective as the *ced-6* homozygotes; no intermediate phenotypes were observed. Duplication losses that led to an engulfment defect are shown by squares; those that had no effect on the engulfment of dying germ cells are indicated by circles. Each symbol represents a single mosaic animal.

the engulfment of cell corpses that underwent apoptosis many hours, or even days, before (Figure 4D). We conclude that there is no specific time window for *ced-6* to act during the process of programmed cell death. Thus, once a cell becomes apoptotic, it appears to be permanently marked for removal.

ced-6 Acts within Engulfing Cells

To determine whether *ced-6* acts within engulfing or dying cells, we performed a classical genetic mosaic analysis (Herman, 1984). For such a study to succeed, the identity of the dying cell and the engulfing cell must be known and the two cells should be well separated in their developmental lineage. The programmed cell deaths that can be observed in the adult hermaphrodite germ line are particularly suitable for such a study. First, the identity of the dying cells (early oocytes) and of the engulfing cells (somatic gonadal sheath cells) is well established (Hengartner, 1997) and invariant. Second, germ cells and gonadal sheath cells arise from distinct parts of the cell lineage (Sulston et al., 1983).

To generate mosaic animals mutant in either the dying germ cells or the engulfing sheath cells, we generated animals of genotype *dpy-17(e164) ced-6(n1813) mec-14(u55) ncl-1(e1865) unc-36(e251) III; sDp3*. These worms carry mutations in *ced-6* and several linked markers. The strain also contains the small free duplication *sDp3*, which carries a wild-type copy of each of the mutated genes present on the chromosome. Animals carrying

the duplication are thus phenotypically wild-type. Free duplications in *C. elegans* are unstable and have a finite chance of being lost during cell division—in the case of *sDp3*, the probability of loss has been estimated at 0.1% per cell division (Yuan and Horvitz, 1990; Hedgecock and Herman, 1995). Loss of the duplication during development leads to mosaic animals in which the cell that lost the duplication and all its descendants are mutant. Loss of the duplication can be assessed by scoring for the cell-autonomously acting gene *ncl-1* (*ncl-1* mutant cells have an enlarged nucleolus, which can readily be scored using DIC optics).

Using this strategy, we identified animals with mutant germ lines but wild-type sheath cells, and vice versa, and assessed them for the presence of persistent cell corpses in the adult gonad (see Experimental Procedures). We found that as long as the sheath cells were wild-type, dying germ cells were efficiently engulfed even if they were mutant for *ced-6*. In contrast, apoptotic germ cells uniformly failed to be engulfed if the sheath cells were mutant for *ced-6* (Figure 5). In several mosaic animals, the duplication was lost only in one of the two somatic precursor cell lineages (Figure 5), resulting in worms with one mutant and one wild-type somatic gonad arm. Interestingly, in these animals, dying germ cells accumulated to high levels in the arm that contained the mutant sheath cells, whereas the *ced-6(+)* arm was wild-type for engulfment. These results demonstrate that *ced-6* acts within the sheath cells, and not the germ cells. Thus, *ced-6* acts within the engulfing cells to promote the efficient removal of apoptotic corpses.

ced-6 Might Act Downstream of *ced-1* and *ced-7*

We took advantage of the *hs::ced-6* transgene that we generated to test whether high levels of *ced-6* expression could compensate for the loss of any of the other known engulfment genes. To this effect, we crossed the *hs::ced-6* transgene into the genetic background of all other engulfment mutants and tested for the presence of persistent cell corpses after heat shock. We found that overexpression of CED-6 significantly suppressed the engulfment defect caused by putative null mutations in *ced-1* and *ced-7* (Ellis et al., 1991; Wu and Horvitz, 1998b [this issue of *Cell*]), suggesting that these genes might act upstream of *ced-6* (Figure 6). In contrast, the *hs::ced-6* transgene had no effect on the number of persistent cell corpses observed in *ced-2* and *ced-5* mutants (Figure 6), suggesting that these genes act either downstream of *ced-6*, or in a distinct genetic pathway. Finally, we observed a weak but statistically significant suppression of the engulfment defect of *ced-10* mutants following CED-6 overexpression (Figure 6; $p < 0.02$, unpaired Student's test). However, because we do not know whether the single available *ced-10* allele is a null mutation, this observation is hard to interpret.

Discussion

The mechanisms that mediate recognition and removal of apoptotic cells are still poorly understood. It has been proposed that dying cells secrete or present on their surface proengulfment signals that, upon contact with

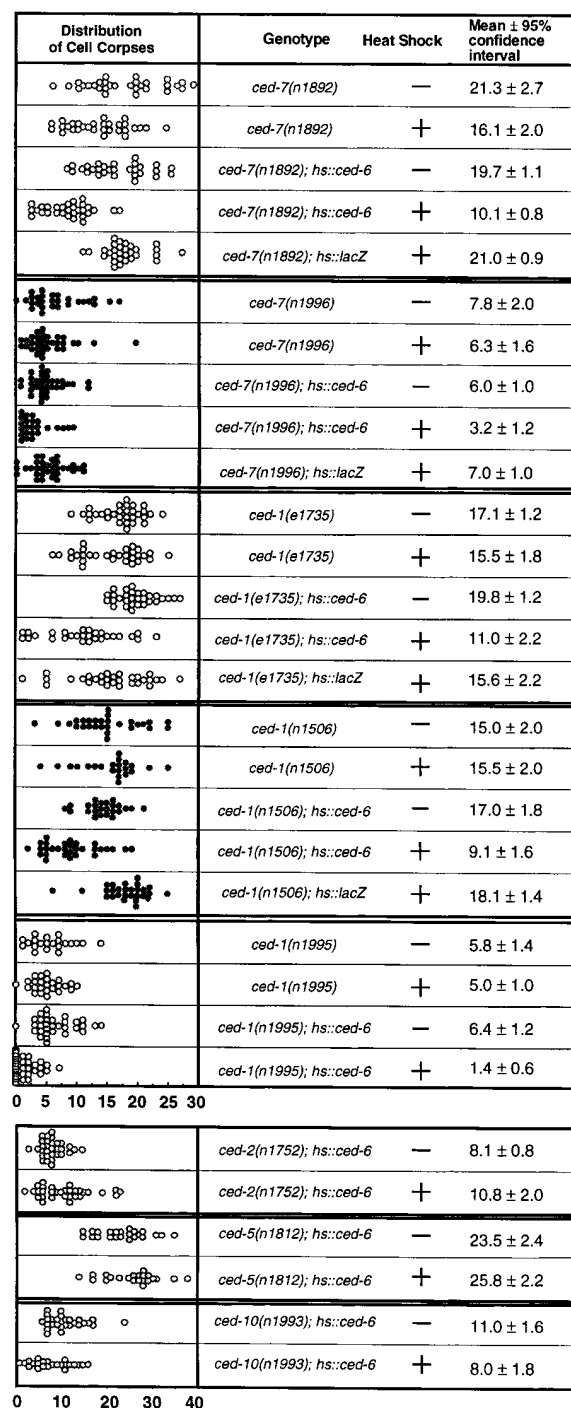


Figure 6. Overexpression of *ced-6* Partially Suppresses the Engulfment Defect of *ced-1* and *ced-7* Mutants

Embryos laid by transgenic mothers were heat shocked just before the wave of embryonic cell death (see Figure 4A) and scored for the number of persistent cell corpses shortly after hatching.

the correct receptors on the phagocytic cell, activate a specific "phagocytic" signal transduction pathway that leads to the engulfment of the apoptotic cell (Savill et al., 1993).

In this present study we have provided evidence that

supports such a mechanism for engulfment of apoptotic cells. We have shown that *ced-6*, whose only known function is to promote the engulfment of apoptotic cells in *C. elegans*, encodes a putative signal transduction protein that acts within the engulfing cell, presumably to recruit the "phagocytosis machinery" that leads to the removal of the neighboring apoptotic cell. Our results start to elucidate a genetic pathway that mediates the engulfment of programmed cell deaths in *C. elegans*.

CED-6 Might Be an Adaptor Molecule Acting in a Tyrosine Kinase Pathway

The most obvious sequence feature of CED-6 is the presence of a PTB domain. Several PTB domain-containing proteins, including Shc and IRS-1 (Yenush and White, 1997), act as adaptor molecules, binding to phosphorylated tyrosine residues present on activated receptors, and recruit downstream molecules to form a signaling complex (Bork and Margolis, 1995; van der Geer and Pawson, 1995; Pawson and Scott, 1997). In addition to the PTB domain, CED-6 contains in its C-terminal half a large number of PxxP signature motifs (Figure 2B). Such motifs, in the right sequence context, have been shown to form a left-handed polyproline type II helix (Feng et al., 1994) that interacts with an SH3 domain (Ren et al., 1993; Yu et al., 1994). The SH3 domain has been proposed to mediate protein-protein interactions between signaling molecules downstream of membrane-bound receptors (Koch et al., 1991; Pawson and Schlessinger, 1993). The presence of a PTB domain and potential SH3-binding sites leads us to suggest that CED-6 is an adaptor protein that directly or indirectly transduces a signal from receptor to effectors or cytoskeletal proteins in order to initiate the engulfment process.

The presence of a PTB domain within CED-6 strongly suggests that transduction of the proengulfment signal within the engulfing cell involves tyrosine phosphorylation. A tyrosine kinase must thus exist to phosphorylate tyrosine residue(s) in a substrate protein that interacts with the CED-6 PTB domain. Could *ced-1* or *ced-7* encode this phosphoprotein? While the molecular nature of *ced-1* has not yet been reported, Wu and Horvitz (1998b) showed that *ced-7* encodes an ABC transporter family member that localizes to the plasma membrane. ABC transporters are known either to import or export ions, small molecules, or even peptides and proteins (Higgins and Gottesman, 1992). Intriguingly, the CED-7 protein contains a YNPLY sequence within one of its predicted cytoplasmic domains (Wu and Horvitz, 1998b). Whether CED-7 could be phosphorylated on this tyrosine residue and recruit CED-6 to the membrane remains to be determined.

CED-6 Acts within Engulfing Cells

We have shown by our genetic mosaic analysis (Figure 5) that CED-6 acts within the engulfing cells to promote the elimination of apoptotic cells. Because of technical constraints, we had limited this analysis to the death of only one specific cell type—the germ cell. However, the results of our heat shock experiments suggest that CED-6 is likely to act within the engulfing cell during

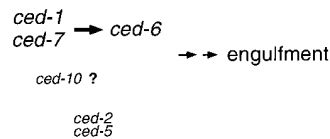


Figure 7. A Genetic Pathway for Engulfment in *C. elegans*

Overexpression of *ced-6* partially suppresses the engulfment defect resulting from loss of *ced-1* or *ced-7* function, suggesting that *ced-6* acts downstream of these genes. Overexpression of *ced-6* has little to no effect on the engulfment defect caused by mutations in *ced-2* and *ced-5*, suggesting that these genes act in parallel to *ced-6*, as previously proposed by Ellis et al. (1991). Alternatively, these genes might act downstream of *ced-6* (not shown). The position of *ced-10* in the pathway is uncertain (see text).

embryonic development. Specifically, we found that expression of *ced-6* promoted the engulfment of persistent cell corpses in embryos, even though these cells had died many hours before the induction of *ced-6* expression. Because cells that have been dead for many hours are very unlikely to maintain the ability to express protein upon induction, we conclude that expression of CED-6 within living, i.e., engulfing cells is sufficient to promote engulfment. Therefore, CED-6 is likely to act within all engulfing cells.

A Genetic Pathway for Apoptosis-Triggered Engulfment in *C. elegans*

ced-6 is but one of over six genes involved in the engulfment of apoptotic cells (Hengartner, 1997). Because mutations in all six genes have similar phenotypes, it has not been possible to determine the order of function of these genes in the engulfment process. We took advantage of the *hs::ced-6* transgene to determine such an order, based on the assumption that high levels of CED-6 protein might generate a weak gain-of-function and as such might suppress mutations in upstream, but not downstream, genes in the *ced-6* pathway. We found that overexpression of *ced-6* partially suppresses the engulfment defect caused by putative null mutations in *ced-1* and *ced-7*. One possible interpretation of these results is that *ced-6* acts downstream of *ced-1* and *ced-7* in the same pathway (Figure 7). The weak suppression of *ced-10* by overexpressed *ced-6* raises the possibility that *ced-10* might also act upstream of *ced-6*. However, more alleles of *ced-10* should be isolated and tested to confirm this hypothesis. The absence of any significant effect of the transgene in *ced-2*, *ced-5* mutant backgrounds suggests that *ced-6* acts either upstream, or independently of these genes (Figure 7).

Evolutionary Conservation of the Pathway for Engulfment

Genetic studies in *C. elegans* have identified over a dozen genes that function in programmed cell death. Several of these genes, such as *ced-3*, *ced-4*, and *ced-9*, have homologs that perform similar function(s) in mammalian apoptosis (Hengartner, 1997), indicating that the apoptotic machinery responsible for cell killing has remained conserved between nematodes and mammals (Hengartner, 1997; Vaux, 1997). The fact that engulfment is a very early event in apoptosis and that its purpose

is to protect surrounding tissue from being damaged suggests that engulfment might have been pressured by evolution into an inevitable part of the death program. Therefore, it might also be conserved.

We have identified a number of ESTs whose predicted translation products show significant sequence similarity to CED-6, both within and outside of the PTB domain (Figure 3). In fact, CED-6 is more similar to several of these EST products than to any previously characterized protein, suggesting that these ESTs might encode direct CED-6 homologs (orthologs), or at least members of the same subfamily of PTB domain-containing proteins. Whether these homologs also function in the engulfment of apoptotic cells is not known.

A strong case for functional conservation can be made for CED-7: Luciani and Chimini (1996) have shown that the mammalian CED-7 homolog ABC1 is expressed on the surface of macrophages and promotes the recognition and clearance of apoptotic cells. Because signal transduction pathways tend to remain conserved through evolution, it is likely that not only CED-7, but also CED-6 and the other *C. elegans* engulfment proteins will have mammalian homologs that participate in the phagocytic removal of apoptotic cells. Studies of the engulfment process in *C. elegans* therefore could provide insight into the molecular basis of apoptotic cell removal in humans.

In addition to their ability to recognize and engulf apoptotic cells, professional phagocytes carry specific surface receptors, such as the Fc (Greenberg et al., 1993; Ravetch, 1994) and C3 (Bianco et al., 1975; Greenberg, 1995) receptors, that recognize antigen-opsonized particles and trigger their phagocytosis. Inhibitor studies have shown that Fc receptor-mediated phagocytosis requires tyrosine phosphorylation (Greenberg et al., 1993; Greenberg, 1995). Our results suggest that the engulfment of apoptotic cells might be also mediated by a tyrosine kinase signal transduction pathway. While these two pathways clearly use distinct receptors at the cell surface, they must eventually converge on the same downstream engulfment machinery and might thus share at least some common signal transduction molecules.

Experimental Procedures

Mutations and Strains

The N2 Bristol strain was used as the reference wild-type strain for this study. All strains were maintained as described by Brenner (1974), except that worms were raised on NGM-lite agar medium (Sun and Lambie, 1997). Strains were maintained and raised at 20°C, unless otherwise noted. The following mutations were used in this study: LG I: *ced-1(e1735, n1995, n1506)*; LG III: *dpy-17(e164)*, *ced-6(n1813, n2095)*, *mec-14(u55)*, *ncl-1(e1865)*, *ced-7(n1892, n1996, n1997)*, *unc-36(e251)*, *sDp3(III, f)* (Rosenbluth et al., 1985); LG IV: *ced-2(e1752)*, *ced-5(n1812)*, *ced-10(n1993)*. Unless otherwise noted, mutations are described in Hodgkin, 1997.

Analysis and Quantitation of Engulfment

Animals were anesthetized with 30 mM NaN₃ and mounted on agar pads for observation using Nomarski optics (Sulston and Horvitz, 1977; Avery and Horvitz, 1987). To quantitate cell corpses generated during embryonic development, we scored the number of persistent cell corpses that were visible in the head region of young L1 larvae (at the 4-cell gonad stage within 6 hr of hatching; Klass et al., 1976). To quantitate the engulfment defect, we counted cell corpses visible within both the distal arm (where the germ cell deaths occur) and the

proximal arm (where persistent germ cell corpses can sometimes be observed as they are swept along by the developing oocytes).

Germ-Line Transformation and Genomic Rescue of *ced-6*

Transgenic animals were generated using the germ-line microinjection procedure developed by Mello and Fire (1995). Cosmids W03A5, F20F10, F48E8, R02F2, W02G12, T06H6, C48E6, C44D7, F56D2, F43F12, C05D2, T06C9, and C05H8 were injected, either singly or in groups (final concentration 20 ng/μl for each cosmid) into *ced-6(n1813)* adult hermaphrodites. We used plasmid pRF4 (final concentration 50–80 ng/μl) as a dominant coinjection marker (Mello and Fire, 1995); pRF4 carries the mutated collagen gene *rol-6(su1006gf)* and confers a dominant roller (Rol) phenotype. Transgenic lines carrying stably transmitting extrachromosomal arrays were kept for further analysis. To assay for rescue, 3-fold embryos laid by transgenic animals were examined for persistent cell corpses under Nomarski optics. Transgenic lines that generated embryos with few or no cell corpses were considered to be rescued. To further define the position of *ced-6* within F56D2, we created a number of deletion constructs and subclones. Of these constructs, 50–90 ng/μl was coinjected with 80–100 ng/μl pRF4 into *ced-6(n1813)* worms, and their rescuing ability was tested as described above. We generated a frameshift mutation at the unique MluI site in the third exon of F56D2.7 by digesting pLQ1 (plasmid containing the XhoI rescuing fragment) with MluI and filling in the 4 bp overhang with Klenow enzyme.

Isolation of *ced-6* cDNAs

To isolate full-length *ced-6* cDNAs, we screened a mixed-stage *C. elegans* cDNA library in λ Zap (gift of R. Barstead, Oklahoma Medical Research Foundation, Oklahoma City, OK) using established protocols (Sambrook et al., 1989). ³²P-labeled probe was made using the rescuing 10 kb XhoI genomic fragment as a template. Positive phage were changed into plasmid clones using an in vivo excision protocol (Stratagene). Clones corresponding to F56D2.7 were identified by Southern blot analysis. For this purpose a ³²P-labeled probe was generated by reverse transcription (RT)-PCR amplification of exons 3–5 of F56D2.7. Primers used for RT-PCR were GAATGTTCTCATTT ATTG and GGATTCACGATCCGATG. From about 300,000 plaques we isolated 10 plasmid clones containing F56D2.7 cDNAs. Two clones with partial SL2 splice leader sequence at their 5' end and intact poly(A) tail were identified as full-length F56D2.7 cDNAs. Sequence and Sau3AI restriction pattern analysis failed to identify any alternative splicing or processing among the 10 clones.

Reverse Transcription-PCR

RT-PCR experiments were performed to determine the 5' end of transcripts detected or predicted to exist within the rescuing XhoI genomic fragment. Reverse transcription was performed with the following primers: C05D2.6A: GAATCTGTCCATCGCATTGC and GAATTCTTTGGGTAGACA; C05D2.6B: GCTCTGAAGAACTGTGA and GACGAGGTGAAGCGATTGTG; F56D2.7: GGGATCAAACGAAT CATC. These primers were then used in combination with SL1 or SL2. Total *C. elegans* mixed-stage RNA was isolated as described by Meyer and Casson (1986). RT-PCR was performed using the Superscript Preamplication System (GIBCO-BRL).

Sequence Analysis

DNA sequences were analyzed and managed using the Geneworks II Program (IntelliGenetics). Database searches were performed using the NCBI BLAST server. Evolutionary relationship among PTB domains was determined using the SeqLab package of the GCG suite, and the tree grown graphically.

Identification of *ced-6* Mutations

To determine whether either mutant *ced-6* allele results in a physically detectable polymorphism, we generated Southern blots of N2, *ced-6(n1813)*, and *ced-6(n2095)* genomic DNA digested with various restriction enzymes. A probe generated from the rescuing XhoI

genomic fragment detected novel allele-specific bands in *ced-6(n2095)* using five different enzymes. Analysis of the novel restriction patterns in *ced-6(n2095)* indicates that this allele carries a complex rearrangement in this region that covers at least part of F56D2.7, but does not affect the neighboring C05D2.6B transcript.

To identify point mutations within F56D2.7, overlapping fragments of the F56D2.7 locus from N2, *ced-6(n1813)*, and *ced-6(n2095)* worms were PCR amplified. These PCR products were directly sequenced using the PCR Product Sequencing Kit (Amersham). The overlapping PCR fragments covered the entire F56D2.7 transcription unit and about 1 kb of upstream genomic sequence. Sequences of the primers used for PCR amplification and sequencing are available upon request.

Heat Shock Experiments

To test whether F56D2.7 cDNA can rescue the *ced-6* engulfment defect, a KpnI/SalI fragment containing the full-length F56D2.7 cDNA clone pLQ33.1 was inserted into KpnI/SacI-digested pPD49.78 and pPD49.83 vectors (Mello and Fire, 1995), which carry *hsp16-2* and *hsp16-41* heatshock promoters, respectively, creating pLQhs1 and pLQhs2. The two constructs (50 ng/μl each) were coinjected with 80 ng/μl pRF4, to generate stably transmitting extrachromosomal arrays. For our control experiments, we used pPD50.2 and pPD50.15, derivatives of pPD49.78 and pPD49.83 in which the heat shock promoters drive the expression of *lacZ* (Fire et al., 1990). Transgenic lines carrying these constructs were generated as described above. Two *hs::ced-6* lines were analyzed in detail (only one line is shown in the figures; similar results were obtained with the other line).

To overexpress *ced-6* before cell death occurs during embryonic development, adult animals were put on a plate seeded with *E. coli* and allowed to lay eggs for 1 hr. Plates were subsequently para-filmed and subjected to heat shock by transfer to a 33°C waterbath for 45 min. Following a 75 min recovery at 20°C, adult animals were removed from the plates. Twelve to fourteen hours after heatshock, hatching L1 larvae (at the 4-cell gonad stage) were scored for corpses in the head region.

To overexpress *ced-6* after the formation of cell corpses during embryonic development, worm plates containing embryos at all developmental stages (but no larvae) were para-filmed and subjected to heat shock in a 33°C waterbath for 45 min. Three hours after the heat shock, freshly hatched L1 larvae were scored for corpses in the head region.

To determine the effect of *ced-6* overexpression on the engulfment of dying germ cells, L4-stage transgenic animals were transferred to new plates and stored at 20°C. Starting 24 hr after the L4/Adult molt, the worms were heat shocked for 45 min at 33°C as described above. Animals were examined for germ cell corpses 12 hr after heat shock. To determine how long the rescue would last (Figure 4C), worms were also scored 18, 24, 36, and 60 hr after heat shock.

To overexpress *ced-6* in the background of other engulfment mutants, *ced-6* or *lacZ*-expressing extrachromosomal arrays were transferred from *ced-6(n1813)* to a wild-type background and then crossed to *ced-1(e1735)*, *ced-1(n1506)*, *ced-1(n1995)*, *ced-7(1892)*, *ced-7(n1996)*, *ced-7(n1997)*, *ced-2(n1752)*, *ced-5(n1812)* and *ced-10(n1993)*, to generate the corresponding transgenic mutant strains. Heat shock experiments were performed as described above.

Genetic Mosaic Analysis

To determine the focus of *ced-6* action in the engulfment of apoptotic germ cells by somatic sheath cells, we constructed the strain *dpy-17(e164) ced-6(n1813) mec-14(u55) ncl-1(e1865) unc-36(e251) III; sDp3*. Animals of this genotype are wild type, as all mutated loci are covered by the *sDp3(III;f)* duplication (Rosenbluth et al., 1985; Yuan and Horvitz, 1990). Spontaneous loss of the duplication during development generates mosaic animals that carry a clone of mutant cells. The focus of action of both *dpy-17* and *unc-36* is in the AB lineage (Yuan and Horvitz, 1990). To identify animals that lost the duplication in the germ line lineage, we thus looked for non-Dpy non-Unc worms that produce only Dpy Unc progeny. From 1000 animals screened, we identified six such mosaics; with the exception of a single animal that had lost the duplication in P1 (which

generates both germ-line and sheath cells), these animals displayed wild-type germ cell engulfment.

We used a direct DIC screen to identify mosaics that had lost *sDp3* in lineages leading to the somatic sheath cells. Out of 900 animals, we identified eight such mosaics. We determined the exact position of duplication loss by scoring cells related by lineage to the sheath and germ cells for the Ncl-1 (enlarged nucleoli) phenotype. For sheath cells, we relied heavily on specific pharyngeal cells; for the germ line, we scored the C and D lineage body wall muscles.

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GenBank Accession Number

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